# Rapid assessment of the sex of codling moth *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) eggs and larvae

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## Abstract

Two different methods were tested to identify the sex of the early developmental stages of the codling moth *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) with a WZ/ZZ (female/male) sex chromosome system. First, it was shown that the sex of all larval stages can be easily determined by the presence or absence of sex chromatin, which is formed by the female-specific W chromosome in interphase nuclei. This trait can also be used to identify the sex of newly hatched larvae but it does require care and accuracy. Secondly, a new sexing technique was developed based on a molecular marker of the codling moth W chromosome. Flanking regions of an earlier described W-specific sequence (CpW2) were isolated and sequenced and a 2.74 kb sequence (CpW2-*Eco*RI), specific for the W chromosome, was obtained. Several PCR tests were conducted, which confirmed that the CpW2-*Eco*RI sequence is a reliable marker for the sex identification in codling moth samples of different geographical origin. In addition, a fragment of a codling moth gene, *period* (*Cpper*) was isolated and sequenced. Results of southern hybridization of the *Cpper* probe with female and male genomic DNA suggested that the *Cpper* gene is located on the Z chromosome. Then a multiplex PCR assay was developed, which co-amplified the CpW2-*Eco*RI sequence to identify the W chromosome and the Z-linked *Cpper* sequence, which served as a positive control of accurate processing of tested samples. The multiplex PCR provides an easy and rapid identification of the sex of embryos and early larval instars of the codling moth.

## Introduction

Methods for identification of the sex in early developmental stages or tissue samples are very helpful in applied fields, such as ecology, conservation biology, animal breeding and production and forensic medicine, etc. The discovery, in the late 1940s, of the Barr body in human female interphase cells enabled sex to be determined and sex-chromosome syndromes to be identified using a simple and straightforward cytogenetic technique based on assessing the presence or absence of the Barr body in a buccal smear (reviewed by Miller 2006). More recently, various techniques have been developed in different organisms, e.g. flow cytometry for rapid identification of sex according to the difference in DNA content based on different sizes of the X and Y or the W and Z sex chromosomes (e.g. Nakamura et al. 1990; Doležel and Göhde 1995). However, the vast majority of the existing methods use PCR

amplification of sex-specific DNA sequences. The best-known example is a universal sex identification test in all non-ratite birds based on PCR detection of two conserved chromo-helicase-DNA-binding (CHD) genes differing in size: the CHD-W gene is located on the W chromosome and thus unique to females, whereas the CHD-Z gene is located on the Z chromosome and occurs in both sexes (Griffiths et al. 1998). A similar method, based on PCR amplification of sequences originating from the X and Y chromosomes, respectively, has been recently developed in the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) (Douglas et al. 2004).

Moths and butterflies (Lepidoptera) have a WZ/ZZ sex chromosome system or its numerical variation, and as in birds, females are the heterogametic sex (reviewed by Traut et al. 2008). In the majority of species, the female-specific W chromosome forms a compact heterochromatin body in interphase nuclei, the so-called W chromatin or sex chromatin, which is an excellent diagnostic marker of the female sex in developed and mature larvae (Traut and Marec 1996).

To date, no molecular technique for sex identification has been developed in Lepidoptera, but there are some sex-linked molecular markers available for a few species. For example, three randomly amplified polymorphic DNA (RAPD) markers, designated W-Kabuki, W-Kamikaze and W-Samurai, are specific to silkworm *Bombyx mori* (Linnaeus) females (Abe et al. 1998). These markers have not been used for sex identification since in this species, phenotypic markers are available. In specially constructed mutant strains, wild-type alleles of autosomal marker genes, coding for visible traits such as an egg colour, larval phenotype or cocoon colour, were translocated onto the W chromosome while their recessive mutant alleles remained located on autosomes. The visible traits exhibited sex-limited inheritance and enabled the separation of males and females according to the sex-specific phenotype during embryonic, larval and pupal development, respectively (Nagaraju 1996).

Recently, two sex-linked molecular markers were developed in the European corn borer *Ostrinia nubilalis* Hübner, the female-specific $(GAAAAT)_n$ microsatellite locus ONW1 (*O. nubilalis* W-chromosome 1) and the Z-located locus ONZ1 (*O. nubilalis* Z-chromosome 1) composed of imperfect 15 nucleotide long repeats $(CAYCARCGTCACTAA)_n$. The markers were used for a population genetic analysis of North American populations of the corn borer (Coates and Hellmich 2003).

Presently, there is an interest to develop techniques for sex identification in early developmental stages of the codling moth, *Cydia pomonella* (Linnaeus), a major pest of pome fruits throughout most of the temperate fruit growing regions in the world (Barnes 1991). Along with other integrated pest management programmes using pesticides and pheromones (Falcon and Huber 1991; Calkins 1998), the sterile insect technique (SIT) is increasingly becoming a component of area-wide integrated pest management (AW-IPM) programmes for suppressing lepidopteran pest populations (Bloem and Bloem 2000). Work is ongoing to develop genetic sexing strains in the codling moth by inserting selectable transgenes into the W chromosome with the aim to produce non-transgenic male-only progeny for sterilization and release (Marec et al. 2005, 2007). It is expected that molecular markers of the W chromosome and methods of sex identification would facilitate mapping these transgenes and testing sex-specific lethality in transgenic lines. In addition, early identification of female eggs and young larvae could facilitate the identification of female-specific genes and promoters to be incorporated in future transformation cassettes.

Available data suggest that the lepidopteran W chromosome is composed mainly of repetitive sequences and transposons (Sahara et al. 2003; Abe et al. 2005; Vítková et al. 2007; Yoshido et al. 2007), and is therefore quite refractory to standard molecular analyses. Recently, a straightforward method to isolate W-chromosome DNA sequences without any previous sequence information was developed (Fuková et al. 2007). With the help of a laser microbeam the sex-chromatin bodies from polyploid cells of codling moth females were isolated. The isolated DNA was used to develop W-chromosome painting probes and to create a W chromosome-specific plasmid library. The subsequent sequence analysis of selected clones revealed two W-specific sequences, CpW2 (acc. no. AM292090) and CpW5 (acc. no. AM292091), but they were of insufficient length for direct utilization as a robust and reproducible sex identification method.

In this study, data on two different approaches are presented to rapidly identify the sex of embryos and young larvae of the codling moth. First, the use of the presence/absence of sex chromatin in interphase nuclei was verified and secondly, flanking regions of the CpW2 clone (see Fuková et al. 2007) were isolated and used as a molecular marker for females. A fragment of a codling moth *period* (*Cpper*) gene was also sequenced. This gene is orthologous to the


J. Appl. Entomol. **133** (2009) 249–261 

Z-linked gene *period* (*per*), originally isolated in the Chinese oak silkmoth *Antheraea pernyi* (Guérin-Méneville) (Reppert et al. 1994; Gotter et al. 1999). A molecular sex-identification technique using multiplex PCR was also developed, which co-amplified a fragment of the extended CpW2 sequence to identify the W chromosome and a partial *Cpper* sequence, which served as a positive control of accurate processing of tested samples.

## Materials and Methods

### Insects

In most experiments, we used two laboratory strains of the codling moth, a strain kept in the laboratory of L.G.N. since 1972 (for its origin and rearing conditions, see Toba and Howell 1991) referred to as the Yakima strain and a strain kept in the laboratory of F.M. referred to as Krym-61 (originating from Krym, Ukraine, but since 1961 kept in the laboratory) (Fuková et al. 2005). Some experiments were also performed with progeny of backcrosses between female hybrids of CpS (*C. pomonella* granulovirus susceptible) and CpR (granulovirus resistant) strains and CpR males (for their origin, see Asser-Kaiser et al. 2007; for rearing method, see Bathon 1981); in this study, we refer to these insects as southern German backcross population (SGBC). We also used several codling moth samples from different geographical regions for PCR sexing using the W-specific molecular marker. Their origin is given in the section Results.

### Preparation of polyploid nuclei

Sex chromatin status can be routinely determined in preparations of highly polyploid nuclei from Malpighian tubules (MPGs) or silk glands of middle-aged to mature larvae and adults (Traut and Marec 1996). Here, we used a modified protocol for preparation of polyploid nuclei from newly hatched and first and second instar larvae.

The larvae were briefly fixed (max. 1 min) in Carnoy's solution (ethanol–chloroform–acetic acid; 6 : 3 : 1) in a black ceramic dish. During fixing, one to two last abdominal segments of the larvae were separated with the help of a pair of tweezers so that the end of gut with associated MPGs was seen attached to the separated segments. The separated part of abdomen was transferred on a slide into a drop (about 20 μl) of 1.5% lactic acetic orcein (LAO) and stained for 2 min. During staining the cuticle of abdominal segments was removed with the help of fine tungsten needles so that only soft tissues remained including the piece of gut and MPGs. After staining, 10–20 μl of LAO were added and the preparation was covered with a coverslip. Immediately, excess LAO was removed by touching the opposite margins of the coverslip with cut pieces of filter paper until the preparation cleared. Finally, the coverslip margins were sealed with nail varnish.

### DNA extractions

To obtain high molecular weight DNA, genomic DNA was extracted separately from females and males, either from adult moths by standard phenol–chloroform procedure (Blin and Stafford 1976) or from fifth instar larvae using the cetyltrimethylammonium bromide (CTAB)-based method (Reineke et al. 1998) with some modifications. Briefly, in the latter method the tissue was ground in 350 μl TES buffer (100 mM Tris–HCl, pH 8.0, 10 mM EDTA, 2% SDS) with 200 ng/μl of proteinase K. Samples were incubated for 1 h at 55–60°C with occasional gentle mixing. The salt concentration was adjusted to 1.4 M by adding 100 μl of 5 M NaCl. Further, 45 μl of 10% CTAB were added, and the mixture was incubated at 65°C for 10 min. Samples were extracted with chloroform–isoamylalcohol (24 : 1), and the water phase was mixed with 150 μl of 5 M ammonium acetate, vortexed and incubated on ice for 30 min. After centrifugation (12 280 ***g*** for 20 min) the supernatants were transferred into new tubes. DNA was precipitated with 0.7 volumes of isopropanol at 4°C overnight. Samples were centrifuged at 12 280 ***g*** for 20 min and pellets were washed with 70% ethanol. DNA was allowed to dry and then dissolved in 50 μl of TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA).

To obtain genomic DNA for PCR from mature larvae or adults (either alive or frozen or fixed in 100% ethanol), individual specimens were homogenized in an appropriate amount of 5% Chelex (500 μl/30 mg tissue) using sterile pestles. After homogenization, proteinase K was added to a final concentration of 0.1 μg/μl, and the tubes were placed at 50–55°C between 2.5 and 6 h. The samples were incubated at 95°C for 10 min and centrifuged at 23 540 ***g*** for 5 min. Supernatant was transferred into a new tube and then stored at −20°C until a sample was used for PCR.

Genomic DNA from individual eggs and young larvae was extracted using a salting out method. The tissue was ground in 15 μl of TNES buffer (50 mM

Tris–HCl, pH 7.5, 400 mM NaCl, 20 mM EDTA, 0.5% SDS) in a microfuge tube. The volume was adjusted to 300 μl of TNES and supplemented with RNase A and proteinase K (final concentration of each was 100 ng/μl). Samples were incubated at 37°C for 1 h and then at 55°C overnight. Each sample was mixed with 85 μl of 5 M NaCl and centrifuged at 12 280 ***g*** for 10 min. Supernatants were transferred into new tubes and DNA was precipitated with one volume of ethanol at −20°C for 1 h. Samples were centrifuged at 12280 ***g*** for 20 min and pellets were washed with 70% ethanol. DNA was allowed to dry and then dissolved in 20 μl of TE buffer.

### Southern hybridization

The procedure of Fuková et al. (2007) was followed. Briefly, CpW2 and CpW2-derived probes for Southern hybridization were generated from insert containing plasmids. Plasmid DNA was labelled with digoxigenin 11-dUTP using DIG DNA Labeling and Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) with universal primers, M13-20 and M13-26, made by Generi Biotech (Hradec Králové, Czech Republic). Codling moth *per* (*Cpper*) probes were generated and labelled by PCR with primers CpoPer92F (5′-ATA GAC TTC GTC CAC CCT TTG-3′) and CpoPer1005R (5′-CTG GAT TTG CTG TCA TTG TAG T-3′) custom-made by Sigma-Genosys (The Woodlands, TX, USA), using codling moth cDNA as a template (see below). Hybridization of genomic DNA, digested with a selected restriction enzyme, with DIG labelled probes overnight at 45°C or 42°C (with the CpW2-*Eco*RI and *Cpper* probes, respectively), followed by stringent washes at 68°C and subsequent chemiluminescent detection, were carried out using reagents from Roche Diagnostics and Pierce (Rockford, IL, USA). Images were recorded with a LAS-3000 Lumi-Imager (Fuji Photo Film Europe GmbH, Düsseldorf, Germany) or FluorChem 8900 (Alpha Innotech Corporation, San Leandro, CA, USA).

### Inverse PCR, cloning and sequencing

Flanking regions of the CpW2 sequence, specific for the codling moth W chromosome, were isolated and sequenced using inverse PCR (IPCR). First, female genomic DNA was digested with *Eco*RI overnight (1 μg DNA in 20 μl reaction) and the resulting fragments were self-ligated with T4 DNA ligase (Takara, Otsu, Japan) at 16°C for 16 h. The sample was purified on G50 column (Amersham Biosciences, Buckinghamshire, UK). Then, 10 μl of the sample were used as a template for IPCR. Primers for IPCR were designed according to the CpW2 sequence (acc. no. AM292090) and custom-made by Generi Biotech. These were: forward 5′-GAA TAT GAA AAT CTC CCT CTC G-3′ and reverse 5′-AAT CAA CAC GGG ATT CTA CAA G-3′. The composition of IPCR mixture was as follows: 1× Ex *Taq* buffer and 2 U TaKaRa Ex *Taq* HS DNA polymerase (Takara), 200 μM of dNTP mix, 500 nM of each primer and 500 ng of template DNA in total volume of 50 μl. IPCR was performed in a Tpersonal Thermocycler (Biometra, Göttingen, Germany) under following conditions: initial denaturation step at 95°C for 2 min, then 30 cycles of 95°C for 20 s, 51°C for 20 s and 68°C for 1 min 20 s. A final extension step was at 68°C for 5 min. DNA fragments were separated on a 1% agarose gel. The band corresponding to the *Eco*RI fragment of CpW2 (its size was known from Southern hybridization experiments; see Fuková et al. 2007) was cut out from the gel, and the DNA was extracted using a QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). Purified IPCR fragments were cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). Clones were screened for the presence of inserts, which correspond to the size of CpW2-*Eco*RI fragment, by *Eco*RI restriction and gel electrophoresis. Selected clones containing inserts of choice were sequenced (BigDye Terminator version 1.1 Cycle Sequencing Kit; Applied Biosystems, Foster City, CA, USA) and analysed by Southern hybridization.

### PCR sexing using W-specific molecular marker

Primers for PCR sexing method were designed according to the sequence of female-specific CpW2-*Eco*RI fragment (acc. no. ET203621). The composition of PCR mixture was as follows: 1× *Taq* buffer and 2 U *Taq* DNA polymerase (Roche Diagnostics), 200 μM of dNTP mix, 500 nM of each primer made by Generi Biotech (CpW2-954F forward: 5′-TTC TCA CAT ACC CCG ATG GT-3′ and CpW2-1354R reverse: 5′-TGC TTT CTC GGG ATA ACG TC-3′; numbers indicate positions in the CpW2-*Eco*RI sequence) and approximately 20 ng template DNA in total volume of 20 μl. The PCR reaction was performed under following conditions: initial denaturation step at 95°C for 3 min, then 35 cycles of 94°C for 20 s, 60°C for 20 s and 72°C for 20 s, and final extension step at 72°C for 3 min. DNA fragments were analysed on a 1% agarose gel.


J. Appl. Entomol. **133** (2009) 249–261 

### Amplification and sequencing of the codling moth *period* (*Cpper*) gene

A primer set for the *Cpper* gene was designed based on the 1046 bp partial mRNA NCBI sequence for codling moth (acc. no. AF063432) (Regier et al. 1998). The primers, custom-made by Sigma-Genosys, were CpoPer269F forward (5′-ACC TTC ATA CCC TTC CTG TTG-3′) and CpoPer621R reverse (5′-TAA AAG ACG ACC ACT CCG TTT-3′). The PCR fragment amplified from the codling moth genomic DNA with this primer set was sequenced.

To obtain a template cDNA for PCR amplification of the *Cpper* probe for Southern hybridization, total RNA was extracted from 2- to 3-day-old adults, 2 h after the initiation of scotophase using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcript PCR (Superscript III One-Step RT-PCR System; Invitrogen) was used to amplify cDNA sequences.

### Multiplexing PCR for the W-chromosome marker and *period* gene

Two compatible sets of primers for use in a multiplexing PCR were identified to serve as a sexing and positive control diagnostic. A 227 bp exon 2 of *Cpper* was used to design primers to amplify a small fragment as the PCR positive control. The primers (Sigma-Genosys) were: CpoPerF1130Bm (F379) (5′-CCA ATG AGG CGA TTG AAG A-3′) and CpoPerR1296Bm (R545) (5′-CTG CCT TGC TTG ACG ATG-3′) (positions in parentheses indicate the location in the original *C. pomonella*, Cp, *period* NCBI sequence, numbers in primer names indicate positions in the *B. mori*, Bm, *period* NCBI sequence). The length of the resulting PCR fragment was 202 bp. Further, the CpW2-954F and CpW2-1354R primers were selected for the amplification of a fragment of the female-specific W-chromosome marker, CpW2-*Eco*RI. The PCR reaction was performed using a real-time Smart Cycler™ System (Cepheid, Sunnyvale, CA, USA) under following conditions: initial denaturation step at 95°C for 30 s, then 45 cycles of 94°C for 20 s, 63°C for 45 s, 72°C for 45 s and final extension step at 72°C for 3 min. DNA fragments were analysed on a 1% agarose gel.

## Results

### Sex discrimination by morphological traits

Although apparent sexual dimorphism is absent in codling moth larvae, there are some sex-specific differences that discriminate between females from males starting the last (fifth) larval instar. Mature male larvae can be recognized by purple-red testes, which are visible through the skin on the dorsal side of the fifth abdominal segment. The testes are less visible during the feeding stage (fig. 1a) because of reddish tinge of ochre-coloured skin, but they are conspicuous during the wandering and pre-pupal stages, and also in diapausing (overwintering) larvae (fig. 1c). The whitish ovaries cannot be seen through the skin of female larvae (fig. 1b). In the pupal stage, the sex can be distinguished under stereomicroscope according to the appearance of genital area on the ventral side of last abdominal segments (cf. fig. 1d,f). Finally, male and female moths can be easily recognized by genitalia at the end of abdomen (fig. 1e,g).

### Female identification by sex chromatin

Sex chromatin in polyploid nuclei is present in codling moth females but absent in males (Fuková et al. 2005; Makee and Tafesh 2007). In the present study, mature larvae of the Krym-61 strain showed a conspicuous sex-chromatin body in oval or lobbed, highly polyploid female nuclei of the MPG cells (fig. 2a,b). The body was clearly seen even at a low magnification. Male nuclei had a uniform appearance of similar-sized chromatin grains without any heterochromatin (fig. 2c). Preparations of the MPGs from middle-aged larvae showed spherical nuclei of a lower ploidy level, nevertheless, the sex-chromatin body could still be seen in female larvae (fig. 2d) but was absent in male larvae (fig. 2e). It should be noted that starting at the third instar, the male sex can be confirmed by dissection of testes, which are colourless in this stage but already form four lobbed vesicles. In newly hatched larvae, the MPGs display small nuclei with granular appearance. In some of these larvae, a small sex chromatin body was identified at a high magnification (100× objective), indicating the female sex (fig. 2f). In other larvae, nuclei were composed of similar-sized chromatin grains (fig. 2g) and these were males.

### Development of a molecular marker of the W chromosome

The W chromosome-specific sequence CpW2, isolated earlier from females of the Krym-61 strain, is only 264 bp long (Fuková et al. 2007). Using IPCR, flanking regions of the CpW2 sequence were isolated and following cloning and sequencing a 2.74 kb

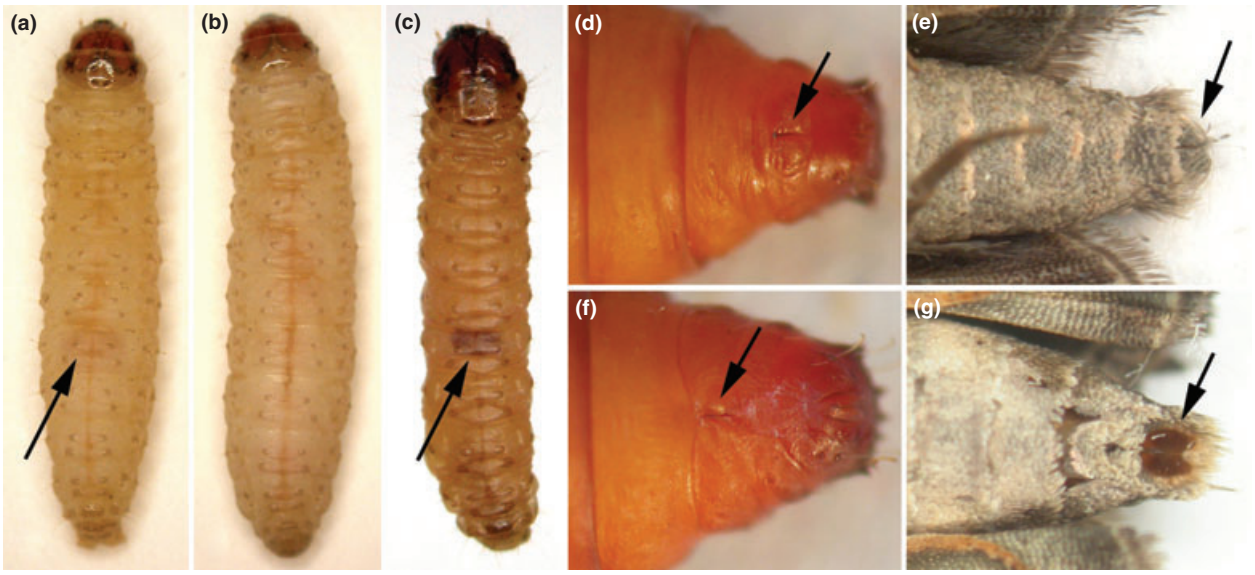


**Fig. 1** Discrimination of codling moth males and females according to morphological traits (arrows) in the last (fifth) instar larvae (a, b, c – dorsal view), pupae (d, f – ventral view) and adults (e, g – ventral view). In the last instar male larvae (a, feeding stage; c, diapausing larva), well-developed purple-red testes are seen through the skin of the fifth abdominal segment, contrary to the female larvae (b). In male pupae (d), two bumps separated by a narrow groove are visible on abdominal segment 9, close to the anus, while the genital area appears as a narrow groove on segment 8 in female pupae (f). In adults, the end of male abdomen (e) displays a pair of valvae, while female abdomen (g) ends with opening, from which the ovipositor is protruded.

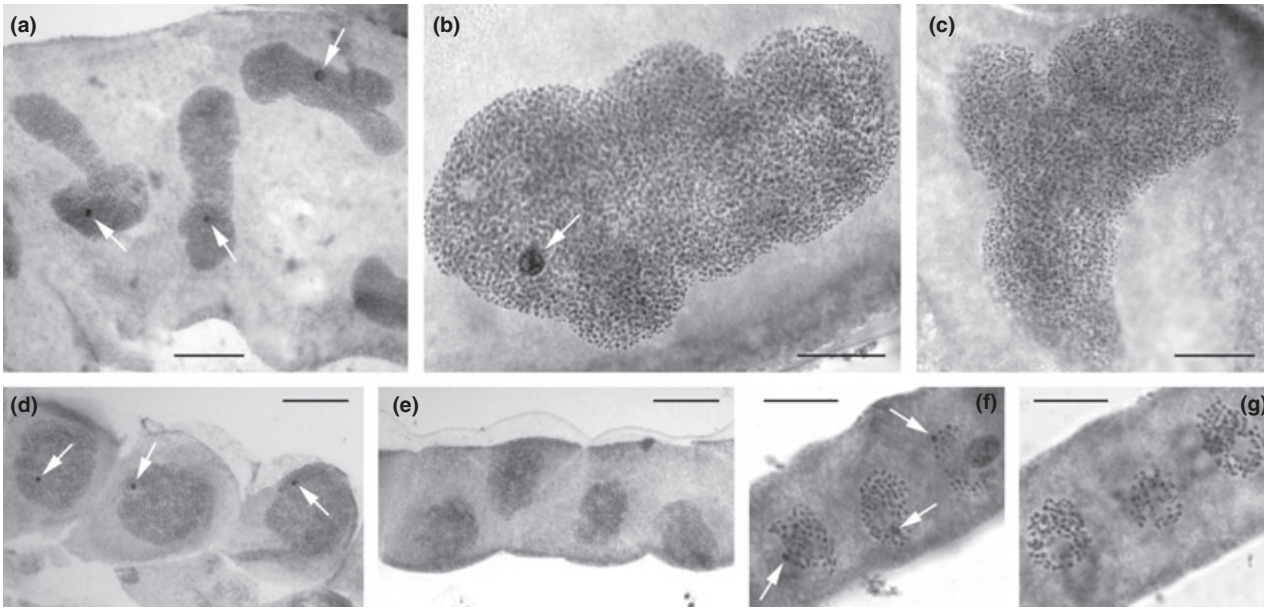


**Fig. 2** Discrimination of codling moth male and female larvae by sex-chromatin body (arrows) seen in polyploid female nuclei (a, b, d, f) of the Malpighian tubules, stained with orcein, but not in male nuclei (c, e, g). (a–c) Fifth instar larvae; (d, e) third instar larvae; (f–g) freshly hatched larvae. Each image shows a piece of a Malpighian tubule with three nuclei except (b) and (c), which show a detail of a highly polyploid nucleus. Bar indicates 50 $\mu$m for (a, d, e), 20 $\mu$m for (b, c) and 10 $\mu$m for (f, g).

sequence was obtained (fig. 3a), designated CpW2-*Eco*RI (acc. no. ET203621). In twice repeated Southern hybridizations, the digoxigenin-labelled CpW2-*Eco*RI sequence hybridized exclusively with female genomic DNA but not with male genomic DNA (fig. 3b). In addition, the hybridization pattern



J. Appl. Entomol. **133** (2009) 249–261 

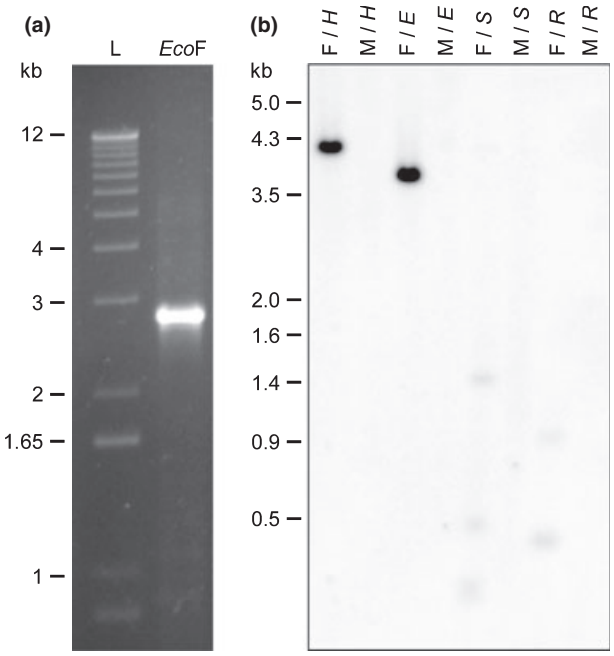


**Fig. 3** (a) Electrophoresis of 2.74 kb CpW2-*Eco*RI fragment (*Eco*F in right lane), isolated by inverse PCR, in a 1% agarose gel stained with ethidium bromide; size specification (left lane, L) was done with 1 Kb Plus DNA Ladder (Invitrogen). (b) Southern hybridization of codling moth female and male genomic DNAs with digoxigenin-labelled CpW2-*Eco*RI fragment; size specification was done with digoxigenin-labelled DNA molecular-weight marker III (Roche Diagnostics); the abbreviations above lanes, F/*H*, M/*H*, F/*E*, M/*E*, F/*S*, M/*S*, F/*R* and M/*R* denote female and male genomic DNA, digested with four restriction enzymes, *Hin*dIII, *Eco*RI, *Sau*3AI and *Rsa*I, respectively.

was similar to that of the CpW2 sequence (see fig. 4 in Fuková et al. 2007). The CpW2-*Eco*RI recognized a single strong band of about 4.2 and 3.8 kb in *Hin*dIII and *Eco*RI digests of female DNA, respectively (the 3.8 kb size of *Eco*RI digest, which was expected to be about 2.74 kb, was most probably distorted by altered mobility of DNA samples and/or digoxigenin-labelled DNA molecular weight marker in the buffer used to separate digested DNAs for Southern hybridization). In *Sau*3AI and *Rsa*I digests of female DNA, three (about 1.4, 0.5 and 0.3 kb) and two (about 0.9 and 0.4 kb) weak bands were observed, reflecting multiple inner restriction sites. The results confirmed that the 2.74 kb CpW2-*Eco*RI fragment is a single- or low-copy number sequence located exclusively on the W chromosome.

### PCR sexing with the W-chromosome molecular marker

The PCR sexing with the CpW2-954F and CpW2-1354R primers designed according to the female-specific CpW2-*Eco*RI sequence was first tested with DNA samples extracted individually from 10 female and 10 male fifth instar larvae of the Krym-61 strain using the CTAB-based method. The sex of the larvae was determined according to the presence/absence of testes visible through the skin and confirmed by dissection. DNA from each female produced a PCR product of 401 bp, corresponding to the part of the

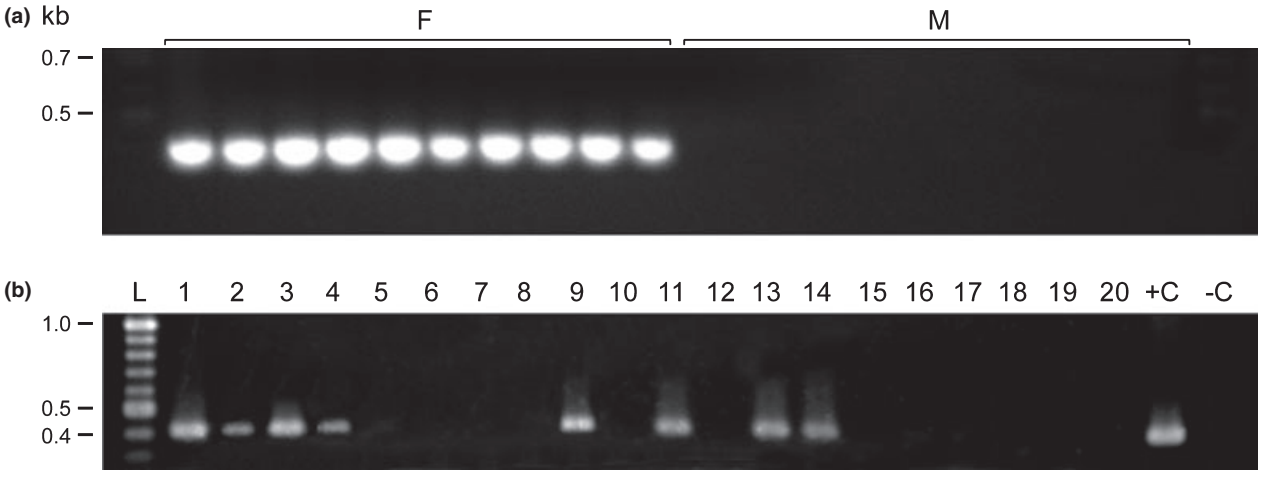


**Fig. 4** Female sex identification based on electrophoresis of PCR products, amplified from genomic DNAs extracted from individual codling moth larvae of the Krym-61 strain. The primers used were designed according to the sequence of female-specific CpW2-*Eco*RI fragment. (a) Control panel showing the corresponding PCR product in left 10 lanes (F), each representing one fifth instar female larva, while no product is seen in right 10 lanes (M), each representing one fifth instar male larva. Size specification was done with mi-100 bp + DNA Marker Go (Metabion, Martinsried, Germany). (b) PCR sexing of 20 hatchlings (lanes 1–20); the presence of PCR product indicates 9 female larvae (lanes 1–4, 9, 11, 13 and 14; sex was confirmed by presence of sex chromatin), the absence 11 male larvae (lanes 5–8, 10, 12 and 15–20; sex was confirmed by absence of sex chromatin); L, DNA ladder; +C, positive control (female DNA); –C, negative control (male DNA). Size specification was done with 1 Kb Plus DNA Ladder (Invitrogen).

CpW2-*Eco*RI sequence determined by the primer pair; whereas no products were obtained from male DNA (fig. 4a).

Next, DNA was extracted individually from 20 newly hatched larvae of the Krym-61 strain by the salting out method. Before extraction, the last abdominal segments of each larva were separated and used for squash preparations to assess the sex chromatin status. Following electrophoresis of PCR products, nine larvae showed a single band of the expected size, while no product was amplified from 11 larvae (fig. 4b). In each larva, the presence of the female specific product correlated with the presence of sex chromatin and its absence with the absence of sex chromatin. Thus, this experiment confirmed the reliability of PCR sexing based on the developed W-specific molecular marker.

A quick PCR sexing test was also performed with DNA samples obtained from seven newly hatched larvae of the SGBC population using the microwave treatment (Goodwin and Lee 1993), i.e. without time-consuming DNA extractions. In the quick test, a clear PCR product of the expected size was obtained in four larvae, indicating the female sex, and the absence of a product in three remaining larvae indicating the male sex (fig. 5).

The above experiments were performed with Krym-61 and SGBC insects. To verify applicability of the PCR sexing test, moths were assayed from two North American populations, from the Yakima strain (fig. 6a) and from the codling moth colony in the mass rearing facility in Osoyoos, British Columbia, Canada (provided by Scott Arthur; fig. 6c). We also tested insects from four other European populations: from Italy and Austria (provided by Peter Neulichedl and Roland Zelger, Laimburg, Italy; fig. 6b), from Switzerland (laboratory strain provided by Silvia Dorn, Zürich; fig. 6c), and from orchards near Chelčice, Czech Republic (diapausing larvae collected and provided by Oldřich Pultar, Biola, Chelčice; fig. 6c). Samples were stored in 100% ethanol, except the diapausing larvae from Chelčice, which were obtained alive. In all samples, each female DNA generated a PCR product of the expected size of about 400 bp, while no product was obtained from male DNA samples (fig. 6a–c). These results suggest that the CpW2-*Eco*RI sequence is well conserved in different codling moth populations and thus is a reliable marker of the female sex.

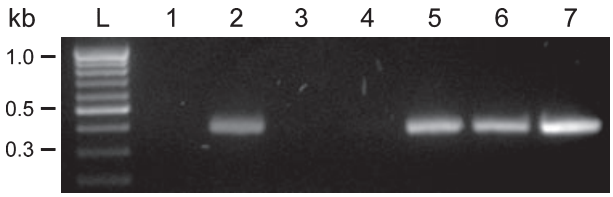


**Fig. 5** Quick PCR sexing test based on electrophoresis of PCR products, amplified from microwave-isolated genomic DNAs of individual codling moth hatchlings of the SGBC population using primers designed according to the sequence of female-specific CpW2-*Eco*RI fragment. The presence of a specific band indicates 4 female hatchlings (lanes 2, 5, 6 and 7), the absence 3 male hatchlings (lanes 1, 3 and 4). Size specification (L, ladder) was done with mi-100 bp + DNA Marker Go (Metabion).

### Codling moth sequence of the *period* (*Cpper*) gene

A 1.5 kb (approx.) product with primers CpoPer269F and CpoPer621R was obtained from codling moth genomic DNA (fig. 7). The size of a PCR fragment amplified from the female cDNA using the same primer set was 394 bp, indicating that there was more than 1 kb of a putative intronic sequence.

The 1.5 kb *Cpper* PCR fragment (acc. no. EU401727) was sequenced and compared to the published cDNA sequence (acc. no. AF063432) (Regier et al. 1998). Two introns were identified and located: intron 1 is 654 bp long and it is inserted between bases 359 and 360 of the cDNA sequence, then exon 2 covers 277 bp, from positions 360–586, and intron 2 is 407 bp long and begins after position 586.

To investigate whether the *Cpper* gene is located on the Z chromosome or on an autosome, Southern hybridization of the *Cpper* probe with equal amount of genomic DNA of females and males digested with different restriction enzymes was performed. After digestion with *Eco*RI and *Xho*I (fig. 8a) and Southern blotting, the *Cpper* probe showed a single band of a high molecular weight, indicating a long restriction fragment, in both female and male DNA (fig. 8b). The band was stronger with male DNA than with female DNA; this was particularly obvious after digestion with *Xho*I. The stronger hybridization of the *Cpper* probe with male DNA was confirmed in an additional Southern hybridization experiment, where DNA was digested either with *Hpa*I or *Mbo*II (fig. 9a,b). With *Hpa*I, a single band corresponding to a restriction fragment of more than 10 kb was obtained. *Mbo*II digestion generated three lower molecular weight fragments because of the two internal restriction sites in the *Cpper* sequence. The stronger hybridization signals with male DNA are compatible with the presence of one copy of the Z chromosome in females and two copies in males.


J. Appl. Entomol. **133** (2009) 249–261 

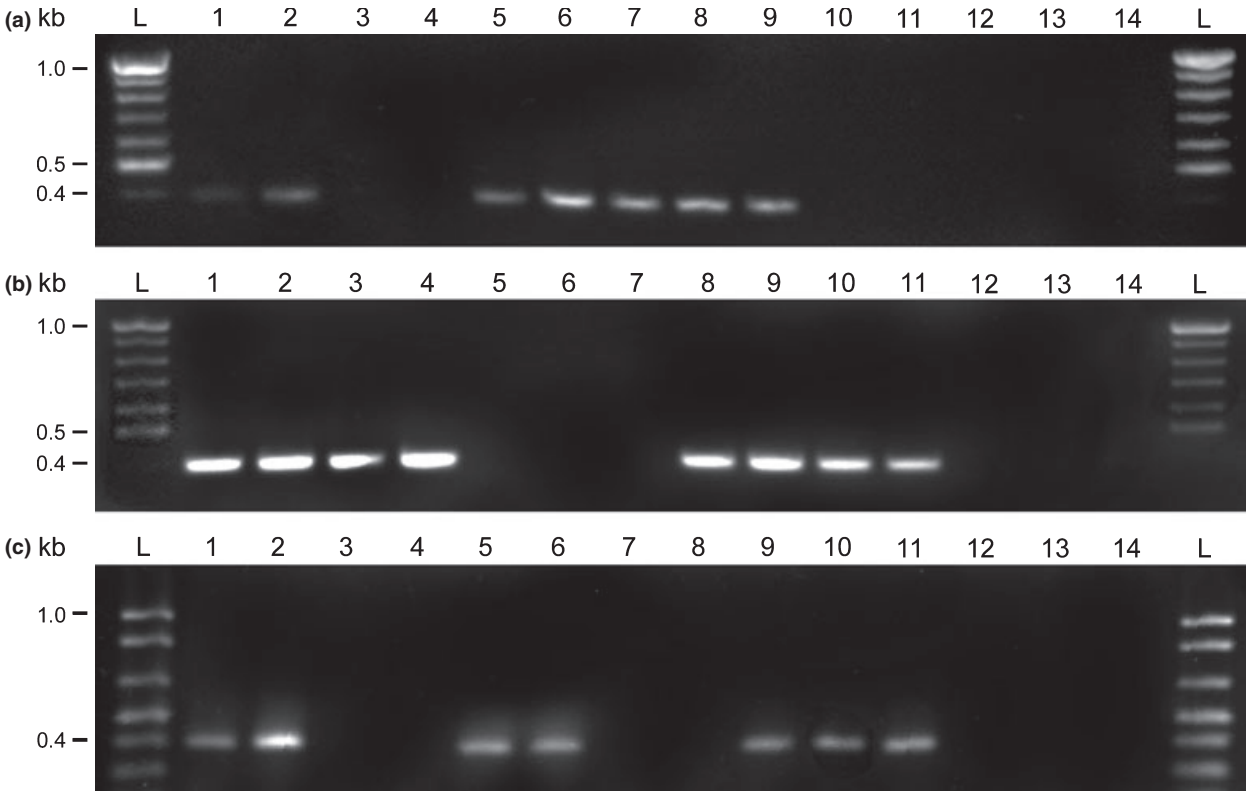


**Fig. 6** Validation of PCR sexing test in codling moth samples of different geographical origin, based on amplification of the 401 bp fragment of the female-specific CpW2-*Eco*RI sequence from genomic DNAs. (a) Yakima strain, U.S.A.: 2 adult females (lanes 1 and 2) and 2 adult males (lanes 3 and 4); 5 female larvae (lanes 5–9) and 5 male larvae (lanes 10–14) of the last instar. (b) Population from Laimburg, South Tyrol, Italy: 4 female larvae (lanes 1–4) and 3 male larvae (lanes 5–7) of the last instar; population from Steiermark, Austria: 4 female larvae (lanes 8–11) and 3 male larvae (lanes 12–14) of the last instar. (c) Zürich strain, Switzerland: 2 adult females (lanes 1 and 2) and 2 adult males (lanes 3 and 4); mass-reared colony from the facility in Osoyoos, British Columbia, Canada: 2 adult females (lanes 5 and 6) and 2 adult males (lanes 7 and 8); diapausing population from Chelčice, Czech Republic: 3 female larvae (lanes 9–11) and 3 male larvae (lanes 12–14). Size specification (L, ladder) was done with mi-100 bp + DNA Marker Go (Metabion) in (a, b) and with 1 Kb Plus DNA Ladder (Invitrogen) in (c).

Thus, the results suggest that the *Cpper* is a Z-linked gene like the *period* gene from *A. pernyi* isolated earlier (Gotter et al. 1999). However, the Southern hybridization data themselves are not a sufficient proof for the Z-linkage of the *Cpper* gene, and its chromosomal location should be verified preferably using fluorescence *in situ* hybridization (FISH).

### Multiplexing PCR for CpW2-*Eco*RI and *Cpper*

A multiplex PCR was conducted, which allowed simultaneous amplification of a fragment of the W-chromosome specific CpW2-*Eco*RI sequence to identify the female sex and the exon 2 fragment of the Z-linked *Cpper* gene, which served us as a positive control. As a template DNA extracted using Chelex from mid to late fourth instar larvae was used (their sex was determined visually by the presence/absence of testes). Amplification of the CpW2-*Eco*RI fragment gave positive results for all females and negative results for all males (fig. 10). The *Cpper* exon 2 fragment was amplified in all samples, indicating that the DNA was of good quality and that the results of the CpW2-*Eco*RI amplification were accurate. The size of the *Cpper* fragment was 202 bp and the size of the amplified CpW2-*Eco*RI fragment was 401 bp.

## Discussion

### Sex chromatin as a cytogenetic marker

Our study confirmed that the sex of middle-aged to mature larvae of the codling moth can be easily determined by routine microscopic preparations, according to the presence or absence of sex chromatin in polyploid interphase nuclei. This trait can also be used to determine sex of newly hatched larvae (even of late embryos). However, sex chromatin in

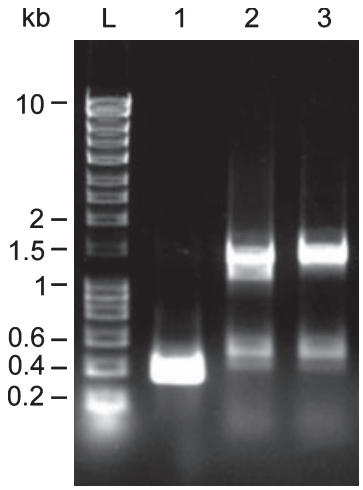


**Fig. 7** Amplification of genomic DNA and cDNA of the codling moth Yakima strain using primers for *Cpper*, CpoPer269F and CpoPer621R. Amplification of female cDNA (lane 1) yielded an approximate 400 bp fragment (expected size was 394 bp). Amplification of female and male genomic DNA (lanes 2 and 3, respectively) yielded a fragment about 1.5 kb long. L, size specification done with Hyperladder I (Bioline, Randolph, MA, USA).

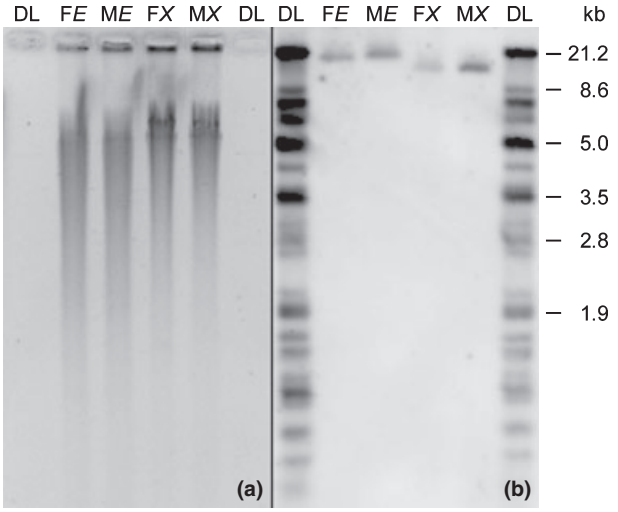


**Fig. 8** Electrophoresis (a) and resulting Southern blot (b) of genomic codling moth DNA with DIG-labelled 956 bp *Cpper* gene fragment obtained by PCR amplification with CpoPer92F and CpoPer1005R primers from female cDNA. The abbreviations above lanes: DL, digoxigenin-labelled DNA molecular-weight marker III + VII (Roche Diagnostics); F*E*, M*E*, F*X* and M*X* denote female (F) and male (M) genomic DNAs (4 $\mu$g per lane), digested with two restriction enzymes, *Eco*RI (*E*) and *Xho*I (*X*) respectively.

the early developmental stages is much smaller because of a low level of polyploidy and its identification requires experience and careful inspection at a high magnification.

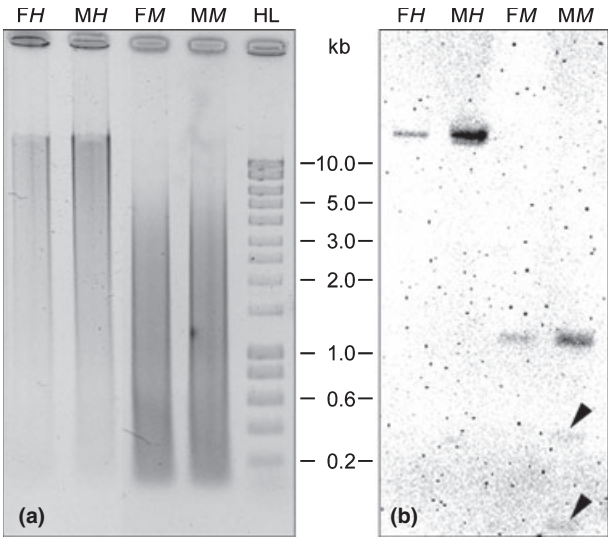


**Fig. 9** Electrophoresis (a) and resulting Southern blot (b) of genomic codling moth DNA with DIG-labelled 956 bp *Cpper* gene fragment obtained by PCR amplification with CpoPer92F and CpoPer1005R primers from female cDNA. The abbreviations above lanes: F*H*, M*H*, F*M* and M*M* denote female (F) and male (M) genomic DNAs (4 $\mu$g per lane), digested with two restriction enzymes, *Hpa*I (*H*) and *Mbo*II (*M*), respectively; arrowheads indicate barely visible bands of the 381 bp (upper) and 81 bp (lower) digests; HL, DNA molecular weight marker Hyperladder I (Bioline).

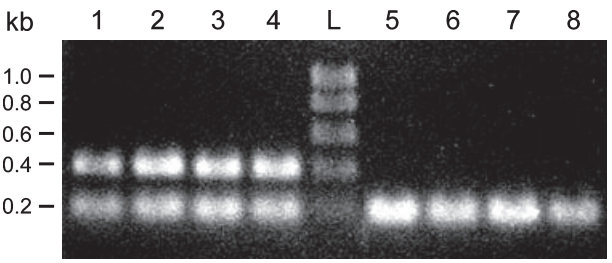


**Fig. 10** Sex identification based on electrophoresis of multiplex PCR products, amplified from genomic DNAs extracted from individual fourth instar codling moth larvae of the Yakima strain with two sets of primers, one for the female-specific sequence (401 bp) of the CpW2-*Eco*RI fragment and the other for the Z-linked sequence (202 bp) of the *Cpper* gene. Female samples (lanes 1–4) show two bands, the upper W-specific band and the lower *Cpper* band. Male samples (lanes 5–8) show only a single *Cpper* band. L, DNA ladder; size specification was done with Hyperladder I (Bioline).

The simple inspection of sex chromatin is almost a universal sexing method in Lepidoptera. It can be used in most species having the W chromosome and long before any morphological marker becomes visible (Traut and Marec 1996). Recently, it was used for sex identification in studies of *Wolbachia* effects on the sex ratio in the adzuki bean borer *Ostrinia scapularis* (Walker) (Kageyama and Traut 2004) and the


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Asian corn borer *Ostrinia furnacalis* (Guenée) (Sakamoto et al. 2007). Sex chromatin also proved to be an excellent cytogenetic marker for other applications such as, screening for radiation-induced sex chromosome aberrations in the flour moth, *Ephestia kuehniella* Zeller (Rathjens 1974), the potato tuber moth, *Phthorimaea operculella* (Zeller) (Makee and Tafesh 2006) and the codling moth (Makee and Tafesh 2007), or in studies on intersexes in the gypsy moth, *Lymantria dispar* (Linnaeus) (Mosbacher 1973).

### Sex identification using molecular markers

A rapid and reliable method for sex determination of the early developmental stages including late embryos and newly hatched larvae in the codling moth has been developed. The sexing method is based on the genomic DNA extraction from individual specimens and subsequent amplification using multiplex PCR with two sets of primers, one set for the detection of the W-chromosome CpW2-*Eco*RI sequence to identify the female sex and the other set for the detection of the codling moth *period* gene (*Cpper*) in both sexes as a positive control. Thus, each female DNA sample should show two bands on the gel, about 200 bp for *Cpper* and about 400 bp for CpW2-*Eco*RI, whereas each male DNA sample only the 200 bp band.

The *Cpper* fragment, used as the control, was designed from exon 2 of the coding region, hence it is expected that this fragment is highly conserved and will be amplified from samples of most codling moth populations. On the other hand, the CpW2-*Eco*RI sequence is a non-coding sequence from the W chromosome, which is formed by heterochromatin and consists mainly of repetitive sequences and transposons (Fuková et al. 2005, 2007). Taken together with the complete absence of meiotic recombination in lepidopteran females (reviewed by Traut et al. 2008) and data suggesting the accelerated molecular divergence of the lepidopteran W chromosomes (Vítková et al. 2007; Yoshido et al. 2007), the CpW2-*Eco*RI sequence might exhibit nucleotide polymorphism in the primer sites and thus, escape PCR amplification. However, during PCR sexing of codling moth samples from different European countries and North America all female DNA produced the female-specific product. This may suggest a common origin of the populations, and it is consistent with relatively recent dispersion of the codling moth around the world (during the past 200 years) following the spread of cultivation of apples and pears (Barnes 1991).

We believe that the method for sex identification is of a great use for many ecological studies and applications including the study of the insecticide resistance. For example, in a recent study on the codling moth resistance to *C. pomonella* granulovirus (CpGV) the sex identification enabled a discovery of the Z-linked inheritance of the CpGV resistance (Asser-Kaiser et al. 2007). The Z-linkage has a very important implication for rapid emergence and spreading of the resistance because of the direct selection acting on females which have only one copy of the Z chromosome and thus one copy of the resistance gene.

The identification of the sex in early developmental stages of the codling moth can greatly improve the search for early expressed genes related to the separation of the sexes. We have been investigating molecular strategies to construct genetic sexing strains to improve the effectiveness of the SIT. One possibility could be to insert an inducible lethal gene, controlled by a constitutive promoter (e.g. the *B. mori actin* promoter), into the W chromosome (Marec et al. 2005). However, the heterochromatic nature of this chromosome may make this strategy impossible or at least very difficult (Marec et al. 2007). An alternative strategy would be to use an early female-specific promoter. To date, no early female-specific genes have been isolated in any tortricid. Nevertheless, using the above system it is now possible to select female eggs for RNA isolation and then search for female specific gene expression and an early female promoter.

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